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Full paper

Dopamine inhibits lipopolysaccharide-induced nitric oxide production through the formation of dopamine quinone in murine microglia BV-2 cells

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ABSTRACT

Dopamine (DA) has been suggested to modulate functions of glial cells including microglial cells. To reveal the regulatory role of DA in microglial function, in the present study, we investigated the effect of DA on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine microglial cell line BV-2. Pretreatment with DA for 24 h concentration-dependently attenuated LPS-induced NO production in BV-2 cells. The inhibitory effect of DA on LPS-induced NO production was not inhibited by SCH-23390 and sulpiride, D_1-like and D_2-like DA receptor antagonists, respectively. In addition, pretreatment with (−)-(6aR,12bR)-4,6,6a,7,8,12b-Hexahydro-7-methylindolo[4,3-a]phenanthridin (CY 208–243) and bromocriptine, D_1-like and D_2-like DA receptor agonists, respectively, did not affect the LPS-induced NO production. N-Acetylcysteine, which inhibits DA oxidation, completely inhibited the effect of DA. Tyrosinase, which catalyzes the oxidation of DA to DA quionone (DAQ), accelerated the inhibitory effect of DA on LPS-induced NO production. These results suggest that DA attenuates LPS-induced NO production through the formation of DAQ in BV-2 cells.

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1. Introduction

Nitiric oxide (NO), a bioactive free radical, is involved in various physiological and pathological processes in many organ systems, including the brain (1, 2). NO is enzymatically formed from arginine by the enzyme NO synthase (NOS). This enzyme exists in 2 forms. One is the constitutive form, which is present in neurons (nNOS) and endothelial cells (eNOS) and is a calcium-dependent enzyme (3, 4). The other is the inducible form (iNOS), which is expressed in various cell types, including microglial cells, in response to a wide variety of stimuli; and it is regulated mainly at the transcriptional level and does not require calcium for its activity (5).

Microglial cells are the resident macrophage-like cells in the central nervous system (CNS). They play critical roles in the immune and inflammatory responses of the CNS (6). Microglial cells in the healthy brain do not express iNOS; but following ischemic, traumatic, neurotoxic or inflammatory damage, they become activated to produce iNOS and to release a large amount of NO (7–10). The excess production of NO causes neuronal apoptosis after acute traumatic spinal cord injury, provokes delayed neuronal death following acute injury in the striatum, and induces delayed neurotoxicity following brain ischemia (11–13). Thus, the regulation of NO production by microglial cells is important for the maintenance of brain homeostasis and is vital for neuronal survival following brain injury.

Dopamine (DA) is the predominant catecholamine neurotransmitter in the mammalian brain and controls a variety of functions, including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. These physiological actions of DA are mainly produced through its interaction with specific G protein-coupled receptors, which are currently recognized as subfamilies of D_1-like and D_2-like receptors (14). On the other hand, DA receptor-independent actions of DA have also been demonstrated. DA readily oxidizes nonenzymatically to DA quinone (DAQ; 15,16), and DAQ can modify cysteine residues of

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certain proteins and modulate their function (17–19). DA is released into the extracellular space via leaky DA synapses or non-junctional DA terminal varicosities and is suggested to modulate not only neuronal functions but also glial cell functions (20). In addition, the release of DA has been shown to increase after brain ischemia (21) and decrease after traumatic brain injury (22). Therefore, it is important to reveal the regulatory role of DA in microglial function in order to understand the pathophysiology of these brain injuries.

In the present study, we investigated the effect of DA on lipopolysaccharide (LPS)-induced NO production in murine microglial cell line BV-2. We firstly demonstrated that DA attenuated NO production in microglial cells through formation of DAQ.

2. Materials and methods

2.1. Antibody and chemicals

Anti-inducible NO synthase (iNOS) polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LPS from *Escherichia coli* (strain O111:B4), DA hydrochloride, the DA D_1-like receptor antagonist SCH-23390, the DA D_2-like receptor antagonist sulpiride, N-acetyl-L-cysteine (NAC), ascorbic acid, nitro blue tetrazolium (NBT), tyrosinase and mouse recombinant tumor necrosis factor-α (TNF-α) were obtained from Sigma (St. Louis, MO, USA). The DA D_1-like receptor agonist (−)-(6aR,12bR)-4,6,6a,7,8,12b-Hexahydro-7-methylindolo[4,3-a]phenanthridin (CY 208-243) was purchased from Tocris Bioscience (Ellisville, MO, USA). The DA D_2-like receptor agonist bromocriptine mesylate, ascorbic acid, hypoxanthine (HX), xanthine oxidase (XO), mouse recombinant interferon-γ (IFN-γ) and all other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Cell culture and treatment with drugs

Cells of the clonal murine microglial cell line BV-2 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS/DMEM), as described previously (Yoshioka *et al.*, 2010). The cells were plated at a density of 1×10^5/well in 24-well tissue culture plates for the Griess assay or at 6×10^5 in 60 mm-diameter dishes for real-time RT-PCR, Western blotting, and NBT/glycinate assay.

Primary cultures of mouse microglia were prepared from postnatal day 0–2 C57BL/6 mice according to the method described by Esen and Kielian (23) with some modifications. In brief, the cerebrum was isolated, minced, and placed in ice-cold 0.25% trypsin-EDTA solution (Sigma–Aldrich, St. Louis, MO, USA). Cells were dissociated for 5 min at 37 °C with agitation every few minutes. After mechanical and chemical dissociation, the population of mixed glial cells was filtered through a-100 μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and plated on 75-cm^2 flasks in FBS/DMEM supplemented with 1% penicillin/streptomycin (Sigma–Aldrich, St. Louis, MO, USA). Mixed glial cultures were maintained in culture in a humidified incubator at 37 °C and 5% CO_2 for 14–20 days, and media were replenished every 3–4 days. Once the primary cultures reached confluence, the microglial cells were isolated from the astroglial cell bed by mechanical agitation on an orbital shaker (200 rpm) for 1 h at 37 °C. The purity of microglial cultures was confirmed with immunostaining for Iba1, and the cultures were >95% pure. The microglial cells were plated in FBS/DMEM supplemented with 1% penicillin/streptomycin at a density of 2×10^5/well in 24-well tissue culture plates for the Griess assay. The animal experiments were performed in accordance with the guidelines of the Japanese Society for Pharmacology and were approved by the Committee for the Ethical Use of Experimental Animals at Setsunan University (approval ID: K12-14). All efforts were made to minimize animal suffering, reduce the number of animals used, and utilize alternatives to *in vivo* techniques.

The cells were incubated with 1–100 μM DA for 1–24 h. Then the culture medium was aspirated and replaced with fresh medium containing LPS or the combination of IFN-γ and TNF-α. To assess the effect of tyrosinase, the treatment of BV-2 cells with tyrosinase was performed in the DMEM without tyrosine and phenol-red (tyrosine-free DMEM). All other experiments were performed using FBS/DMEM. Surpiride, CY208-243 and bromocriptine were dissolved in dimethyl sulfoxide and diluted in FBS/DMEM. SCH-23390, sulpiride, NAC and ascorbic acid were added 1 h before exposure to DA.

2.3. Griess assay

For verification of NO production, the level of nitrite, a stable end product of NO metabolism, was measured in cell-free supernatants by using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid), as described previously (24). Briefly, after LPS treatment, cell-free supernatants were obtained and mixed with an equal volume of the Griess reagent. The absorbance was measured at 570 nm with a microplate reader (Model 680, BIO-RAD, Hercules, CA, USA), and nitrite concentrations were determined with reference to a calibration curve prepared with sodium nitrite standards.

2.4. RT-PCR and real-time RT-PCR

Total RNA was isolated from cells by using a SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA (1 μg each) was reverse transcribed with random primers (Invitrogen, Grand Island, NY, USA) and M-MLV reverse transcriptase (Invitrogen).

The primer sequences for the RT-PCR analysis of dopamine receptor subtypes were D_1 (sense: 5′-AACTGTATGGTGCCCTTCTGTGG-3′, antisense: 5′-GCCCCGTTGTTGTTGATGCTTAC-3′), D_2 (sense: 5′-CACTCCGCCACTTCTTGACATACA-3′, antisense: 5′-AGCCCCATCCA-CAGCCTCCTCT-3′), D_3 (sense: 5′-GTCCTGCCCTCTCCTCTTTGGTTT-3′, antisense: 5′-CATTTGTCCCGTGGCATCTGA-3′), D_4 (sense: 5′-TGCCCTCAACCCCATCATCTACAC-3′, antisense: 5′-TCCCAACCCC-CAGCCTTCATAA-3′) and D_5 (sense: 5′-GGGAGATCGCTGCTGCC TATGTC-3′, antisense: 5′-ATTGGGGGTGAGTGGTGAGATTTT-3′). Thermal cycling conditions included 2 min at 55 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min. The PCR product was subjected to electrophoresis on 2% agarose, stained with ethidium bromide, and viewed under UV light.

Real-time PCR was carried out by using SYBR Green fluorescence according to the manufacturer's instructions (ABI Prism 7900-HT, Applied Biosystems, Foster City, CA, USA). The C_p value of iNOS (sense: 5′-ACATCGACCCGTCCACAGTAT-3′, antisense: 5′-CAGAGG GGTAGGCTTGTCTC-3′, 177 bp) was normalized based on that of β-actin (sense: 5′-AGTGTGACGTTGACATCCGTA-3′, antisense: 5′-GCCAGAGCAGTAATCTCCTTCT-3′, 112 bp). A melting curve analysis was carried out after amplification to verify the accuracy of the amplicon formation.

2.5. Western blot analysis

To prepare cell lysates for immunodetection of iNOS, we lysed BV-2 cells in a buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM sodium chloride, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 10 μg mL^{-1} aprotinin, and 100 μM phenylmethylsulfonyl fluoride (PMSF). The cell lysate (30 μg protein) was blotted onto a polyvinylidene difluoride membrane after separation of the proteins by SDS-PAGE. The membrane was stained with Ponceau S to confirm equal loading and transfer

efficiency. The membrane was then rinsed 3 times with Tris-buffered saline (TBS) and blocked for 30 min with 1% non-fat dry milk/TBS/0.1% Tween-20. It was next incubated for 2 h at room temperature with the anti-iNOS polyclonal antibody (1:1000 dilution). Thereafter the membrane was rinsed 5 times with TBS/0.1% Tween-20, and subsequently incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG polyclonal antibody (Cappel, West Chester, PA, USA). Signals were detected with a chemiluminescence detection kit (NEN Life Science Products, Boston, MA, USA).

2.6. NBT/glycinate assay

For detection of protein-bound quinones (quinoprotein), the NBT/glycinate assay was performed as described by Paz *et al.* (25) with some modification. Total cell lysates were prepared with ice-cold buffer containing 10 mM sodium phosphate (pH 7.4), 138 mM sodium chloride, 2.7 mM potassium chloride, 100 μM PMSF, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. Each cell lysate (400 μg protein/200 μL) was added to 1 mL of NBT reagent (0.24 mM NBT in 2 M potassium glycinate, pH 10.0), and the mixture was then incubated in the dark for 2 h on a shaker. The absorbance of the blue-purple color developed in the reaction mixture was measured at 530 nm.

2.7. Statistical evaluation

The results were expressed as the means ± s.e. obtained from 3 to 4 independent experiments. One-way ANOVA was used to test for differences between group means. When appropriate, *post hoc* multiple comparisons were performed to test for differences between experimental groups (Scheffe's test or Dunnett's test). When the *P* value was less than 0.05, the difference was considered to be significant.

3. Results

3.1. DA attenuated LPS-induced NO production and iNOS expression in murine microglial BV-2 cells

We estimated LPS-induced NO production in BV-2 cells using the Griess assay. Treatment of the cells with LPS for 24 h increased NO production in a concentration-dependent manner (Fig. 1A). We examined the effect of pretreatment of the cells with DA on the LPS-induced NO production. Pretreatment of the cells with DA for 24 h attenuated the LPS-induced NO production in a concentration-dependent manner (Fig. 1B). DA at a concentration up to 100 μM did not have any cytotoxicity towards BV-2 cells (data not shown). The time-course of 10 μM DA pretreatment revealed that a minimum of 9 h pretreatment was required to inhibit the LPS-induced NO production (Fig. 1C). Next, we examined the effect of pretreatment with DA for 24 h on the levels of iNOS mRNA and protein. The pretreatment with DA inhibited the expression of iNOS mRNA and protein induced by LPS in a concentration-dependent manner (Fig. 1D–F). These results suggest that DA attenuated LPS-induced NO production by inhibiting the induction of iNOS protein in BV-2 cells.

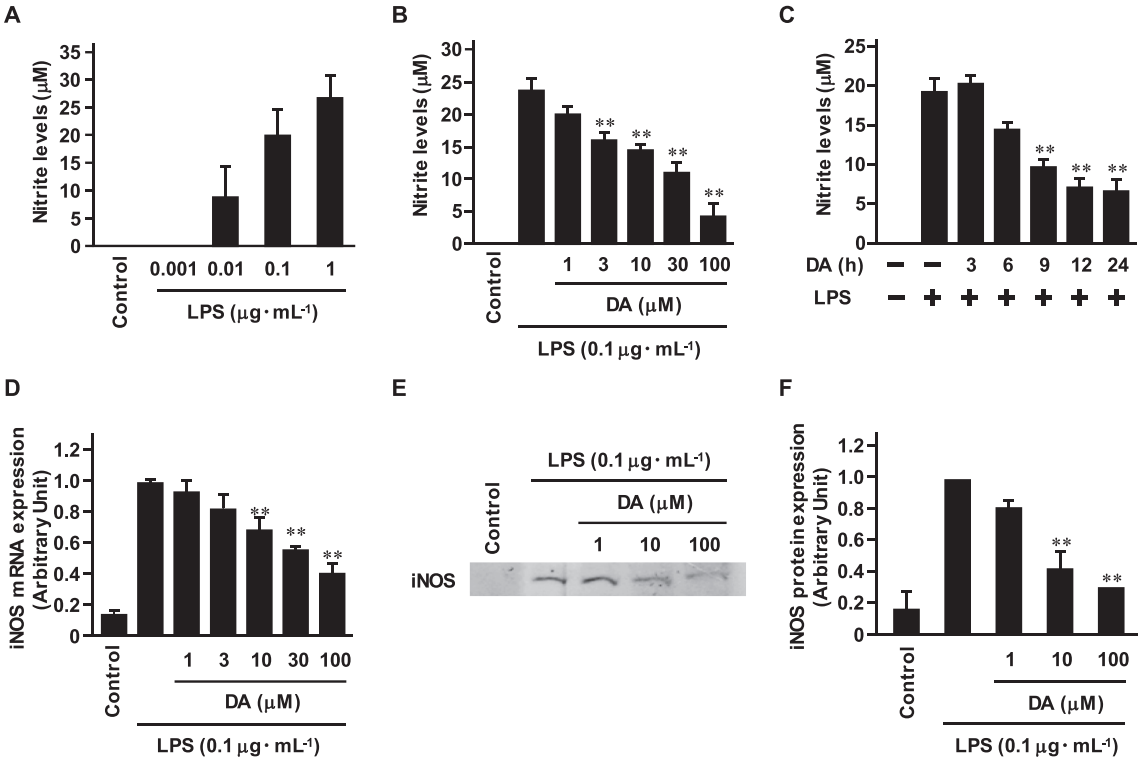


Fig. 1. DA attenuated LPS-induced NO production by inhibiting the induction of iNOS in microglial BV-2 cells. (A) BV-2 cells were treated with LPS at the indicated concentrations for 24 h. The level of nitrite was then determined by performing the Griess assay. (B–F) BV-2 cells were treated with DA at the indicated concentrations for 24 h (B, D, E), or treated with 10 μM DA for indicated times (C). Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The levels of nitrite (B, C), iNOS mRNA (D), and iNOS protein (E) were subsequently determined by performing the Griess assay, real-time RT-PCR and Western blotting, respectively. Relative intensity of iNOS protein band was quantitated by using a densitometer. The levels of iNOS mRNA (D) and protein (F) were expressed as relative to the maximum expression level, which was arbitrarily set as 1.0. Results show the mean ± s.e. obtained from 3 to 4 independent experiments (A–D, F) and blots representative of 4 independent experiments (E). $^{**}P < 0.01$, significantly different from values without DA pretreatment (Sheffe's test).

3.2. Attenuation of LPS-induced NO production by DA was independent of DA receptor activation

We examined the expression of D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) DA receptors by RT-PCR in BV-2 cells and mouse primary microglial cells, and confirmed the expression of D_1-like and D_2-like receptors in BV-2 (D_2 and D_5) and primary microglial cells (D_1 and D_2, Fig. 2). Neither D_3 nor D_4 receptor mRNA was detected in BV-2 and primary microglial cells. We investigated the involvement of DA receptors in the attenuation of LPS-induced NO production by DA. D_1-like DA receptor antagonist SCH-23390 and D_2-like DA receptor antagonist sulpiride did not affect the attenuation of LPS-induced NO production by 10 μM DA (Fig. 3A). In addition, pretreatment with D_1-like DA receptor agonist CY 208−243 and D_2-like DA receptor agonist bromocriptine for 24 h did not attenuate LPS-induced NO production (Fig. 3B). Pretreatment with the combination of 10 μM CY 208-243 and 10 μM bromocriptine also did not attenuate LPS-induced NO production. These results suggest that the inhibition of LPS-induced NO production by DA was independent of DA receptors.

3.3. DA attenuated LPS-induced NO production through the formation of DAQ

We examined the effects of NAC and ascorbic acid on the attenuation of LPS-induced NO production by DA to investigate the involvement of ROS and DAQ in the effect of DA. The attenuation of LPS-induced NO production by pretreatment with 10 μM DA was completely inhibited by 10 mM NAC or 500 μM ascorbic acid (Fig. 4A). On the other hand, pretreatment of the cells with the combination of XO (1−30 mU/mL) and HX (50 μM), which generates superoxide anion, did not affect the LPS-induced NO production (Fig. 4B), nor did that with hydrogen peroxide (10−100 μM, Fig. 4C). These results suggest that the DAQ was involved in DA-induced inhibition of NO production.

To assess the involvement of DAQ in the inhibitory effect of DA on NO production, we examined the effect of tyrosinase, which catalyzes the oxidation of DA to DAQ (26), on DA-induced attenuation of LPS-induced NO production. To avoid the effect of ROS,

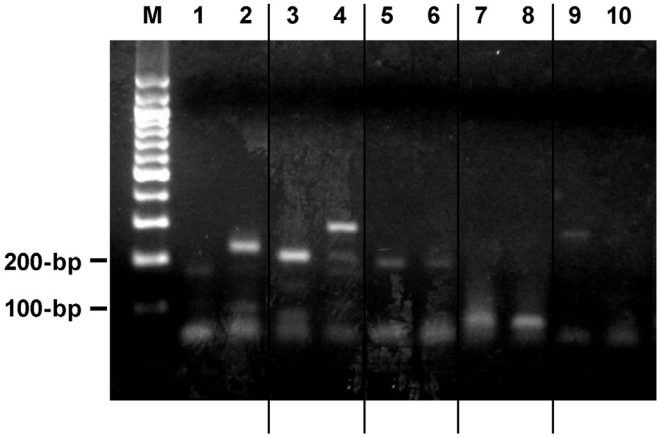


Fig. 2. RNA expression of dopamine receptor subunits in BV-2 cells and mouse primary microglial cells. The mRNA expressions of D_1-like and D_2-like dopamine receptors in BV-2 cells and mouse primary microglial cells were examined by RT-PCR analysis. Agarose gel electrophoresis of PCR amplified products of D_1 (230 bp; lane 1 and 2), D_2 (208 bp; lane 3 and 4), D_3 (310 bp; lane 5 and 6), D_4 (202 bp; lane 7 and 8), and D_5 (221 bp; lane 9 and 10) DA receptors of BV-2 cells (lane 1, 3, 5, 7 and 9) and mouse primary microglial cells (lane 2, 4, 6, 8 and 10) is shown. Lane M represents 100-bp DNA ladder marker. Results show representative results of 4 independent experiments.

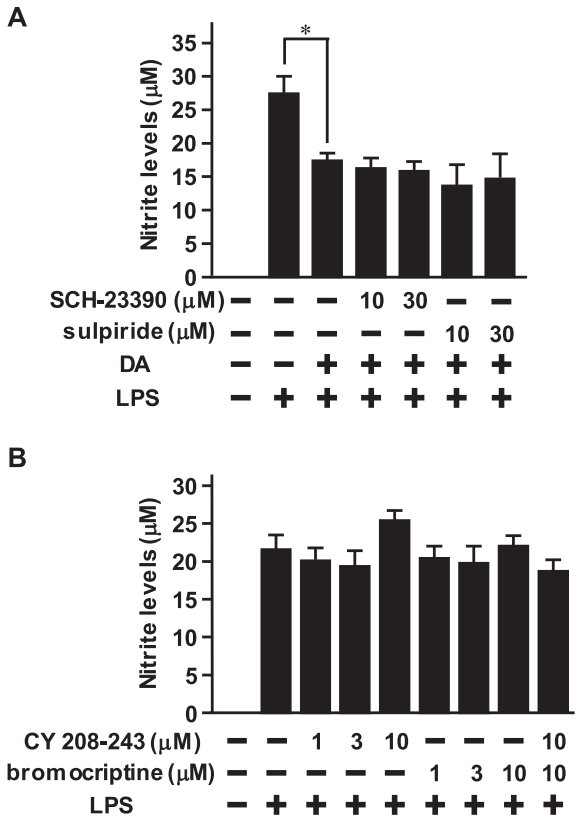


Fig. 3. DA receptor-independent inhibition by DA of LPS-induced NO production in BV-2 cells. (A) BV-2 cells were treated for 24 h with 10 μM DA in the absence or presence of SCH-23390 (10, 30 μM) or sulpiride (10, 30 μM). Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. (B) BV-2 cells were treated with CY 208-243 and/or bromocriptine at the indicated concentrations for 24 h. Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 4 independent experiments. *$P < 0.05$, significant difference between bracketed values (Sheffe's test).

which are produced in the process of tyrosine oxidation by tyrosinase, we estimated the effect of tyrosinase in tyrosine-free DMEM without FBS. In the absence of tyrosinase, the pretreatment with 10 μM DA for 1 h did not affect LPS-induced NO production (Fig. 4D). On the other hand, in the presence of 300 U/mL tyrosinase, the pretreatment with 10 μM DA for 1 h significantly attenuated LPS-induced NO production.

Next, we examined the formation of quinoprotein after DA treatment. Treatment with DA for 24 h concentration-dependently increased the level of quinoprotein (Fig. 5A), and this increase was significantly inhibited by 10 mM NAC or 500 μM ascorbic acid (Fig. 5B). In the absence of tyrosinase, the pretreatment with 10 μM DA for 1 h did not affect the level of quinoprotein (Fig. 5C). On the other hand, in the presence of 300 U/mL tyrosinase, treatment with 10 μM DA for 1 h significantly increased the level of quinoprotein.

3.4. DA attenuated NO production induced by the combination of IFN-γ and TNF-α in BV-2 cells

As it has been reported that cytokines such as IFN-γ and TNF-α induce NO production in microglial cells (27), we examined the effect of DA on cytokine-induced NO production in BV-2 cells. Treatment of the cells with TNF-α concentration-dependently increased NO production in the presence of 3 U mL^{-1} IFN-γ but not in the absence of it (Fig. 6A). Pretreatment with DA for 24 h

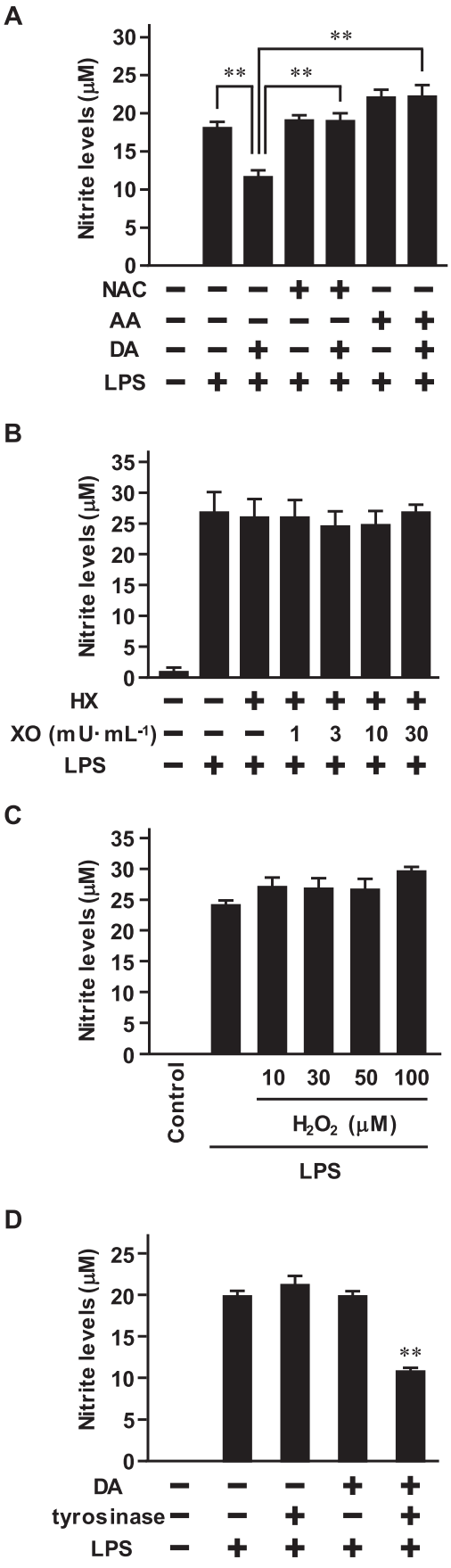


Fig. 4. DA attenuated LPS-induced NO production through the formation of DAQ in BV-2 cells. (A) BV-2 cells were treated for 24 h with 10 μM DA in the absence or presence of 10 mM NAC or 500 μM ascorbic acid (AA). Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significant difference between bracketed values (Dunnett's test). (B, C) BV-2 cells were treated with 50 μM HX alone or in combination with it and XO (B) or with hydrogen peroxide (C) at the indicated concentrations for 24 h. Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 3 independent experiments. (D) BV-2 cells were treated for 1 h with 10 μM DA in the absence or presence of 300 U mL^{-1} tyrosinase in tyrosine-free DMEM without serum. Then the cells were treated with 0.1 μg mL^{-1} LPS in FBS/DMEM for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significantly different from values without DA pretreatment (Sheffe's test).

attenuated the NO production induced by the combination of 10 ng mL^{-1} TNF-α and 3 U mL^{-1} IFN-γ in a concentration-dependent manner (Fig. 6B), and this attenuation was negated by 10 mM NAC (Fig. 6C).

3.5. DA attenuated LPS-induced NO production in mouse primary microglial cells

We examined the effect of DA on LPS-induced NO production by mouse microglial cells in primary culture. Pretreatment of the cells with DA for 24 h attenuated the LPS-induced NO production in a concentration-dependent manner (Fig. 7A). DA at a concentration up to 100 μM did not have any cytotoxicity towards the mouse primary microglial cells (data not shown). The attenuation of LPS-induced NO production by pretreatment with 10 μM DA was partially inhibited by 10 μM sulpiride, but not by 10 μM SCH-23390 (Fig. 7B). Also, the LPS-induced NO production was significantly attenuated by pretreatment with 10 μM bromoctiptine, but not with 10 μM CY208-243 (Fig. 7C). The attenuation of LPS-induced NO production by pretreatment with 10 μM DA was partially, but significantly, inhibited by 10 mM NAC (Fig. 7D).

4. Discussion

Activated microglia secrete an excess amount of NO, which has been implicated in the induction of neuronal damage in certain types of brain injury. Therefore, the regulation of NO production by microglial cells is important for the maintenance of brain homeostasis and is vital for neuronal survival following brain injury.

It has been reported that many physiological actions of DA are mainly mediated by its receptors (14). In the present study, neither SCH-23390 nor sulpiride, D_1-like and D_2-like receptor antagonists, respectively, affected the attenuation of LPS-induced NO production by DA in BV-2 cells (Fig. 3A). In addition, pretreatment with neither CY 208-243 nor bromocriptine, D_1-like and D_2-like receptor agonists, respectively, inhibited this production (Fig. 3B). These results indicate that DA inhibited the LPS-induced NO production independently of DA receptors in BV-2 cells. It was reported that DA attenuated LPS-induced NO production through both D_1-like and D_2-like receptors in mouse and rat microglial cells in primary culture (28). In this study, in mouse primary microglial cells, the LPS-induced NO production was significantly attenuated by bromoctiptine, and the attenuation of LPS-induced NO production by pretreatment with DA was partially inhibited by sulpiride (Fig. 7B and C). In addition, although NAC nearly completely inhibited the effect of DA in BV-2 cells (Fig. 4A), NAC partially inhibited its effect in mouse microglial cells in primary culture (Fig. 7D). Therefore, DA may have attenuated LPS-induced NO production by microglial cells through both DA receptor-dependent and -independent

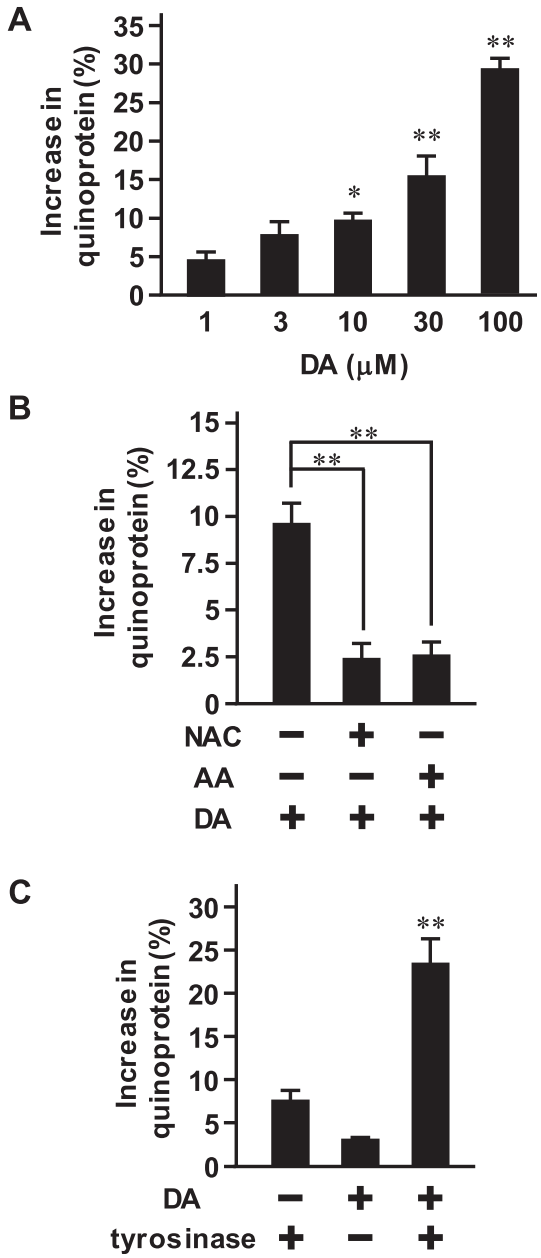


Fig. 5. Effects of NAC and ascorbic acid on DA-induced increase in the level of quinoprotein in BV-2 cells. (A) BV-2 cells were treated with DA at the indicated concentrations for 24 h. Then the quinoprotein level was determined by performing the NBT/glycinate assay. The quinoprotein level was expressed as percentage increase over that in the untreated control. Results show the mean ± s.e. obtained from 4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from DA-untreated control (Sheffe's test). (B) BV-2 cells were treated for 24 h with 10 μM DA in the absence or presence of 10 mM NAC or 500 μM ascorbic acid (AA). Then the quinoprotein level was determined by performing the NBT/glycinate assay. The quinoprotein level was expressed as percentage increase over that in the untreated control. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significant difference between bracketed values (Dunnett's test). (C) BV-2 cells were treated for 1 h with 10 μM DA in the absence or presence of 300 U mL^{-1} tyrosinase in tyrosine-free DMEM without serum. The quinoprotein level was expressed as percentage increase over that in the untreated control. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significantly different from the untreated control (Sheffe's test).

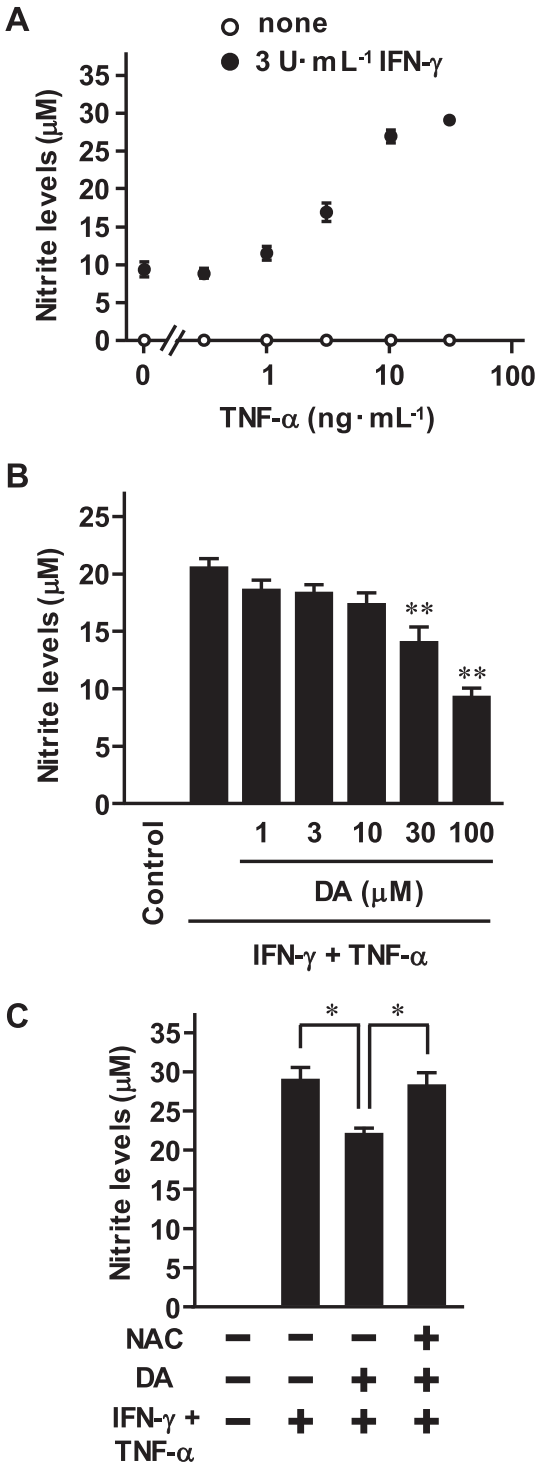
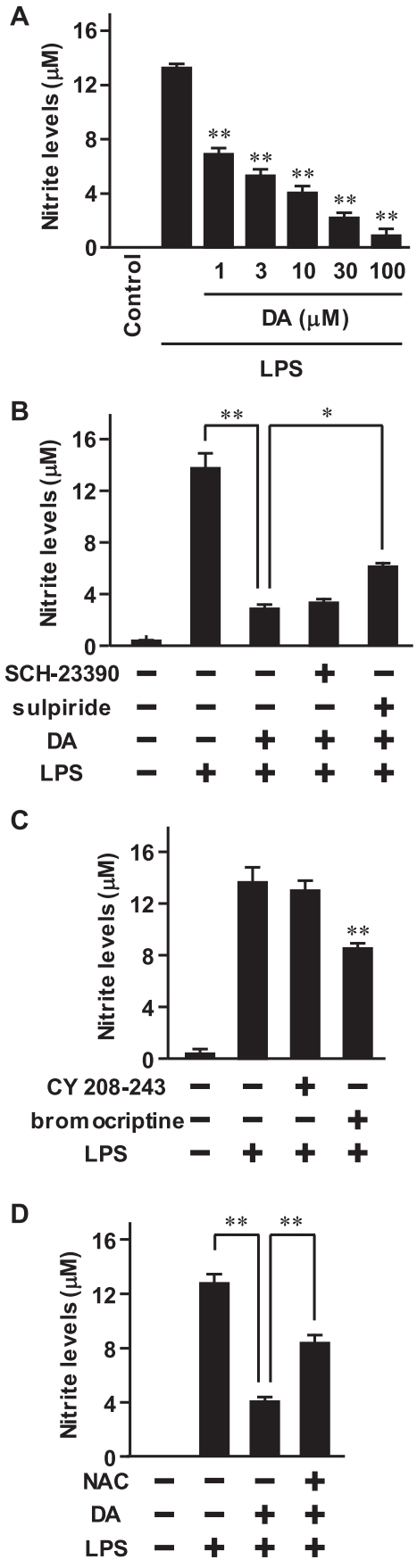


Fig. 6. DA attenuated NO production induced by the combination of IFN-γ and TNF-α in BV-2 cells. (A) BV-2 cells were treated for 24 h with TNF-α at the indicated concentrations in the absence (open circle) or presence (closed circle) of 3 U mL^{-1} IFN-γ. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 3 independent experiments. (B) BV-2 cells were treated with DA at the indicated concentrations for 24 h. Then the cells were treated with a combination of 3 U mL^{-1} IFN-γ and 10 ng mL^{-1} TNF-α for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significantly different from 3 U mL^{-1} IFN-γ and 10 ng mL^{-1} TNF-α (Sheffe's test). (C) BV-2 cells were treated for 24 h with 30 μM DA in the absence or presence of 10 mM NAC. Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significant difference between bracketed values (Dunnett's test).

mechanisms. In this study, although we could not find differences in mRNA expression of D_2-like DA receptor subtypes between mouse primary microglial cells and BV-2 cells (Fig. 2), the LPS-induced NO production was significantly attenuated by



bromoctiptine in mouse primary microglial cells (Fig. 7C), but not in BV-2 cells (Fig. 3B). This discrepancy may be due to the difference in the magnitude or the pathways of postreceptor signaling after D_2 receptor stimulation in each cell. Further studies are needed to reveal this discrepancy concerning DA receptor-mediated action of DA on NO production.

It is well known that DA oxidation generates ROS such as hydrogen peroxide and superoxide anion (15). It was earlier demonstrated that hydrogen peroxide inhibits IL-1-induced NO production in rat mesangial cells (29) and that superoxide anion generated by autoxidation of L-dopa inhibits LPS/INF-γ-induced NO production in rat C6 glioma cells (32). In the present study, pretreatment with hydrogen peroxide or superoxide anion did not affect the LPS-induced NO production (Fig. 4B and C). The differences between these reports and our study may be explained by the difference in cell types. It has been reported that DA readily oxidized to DAQ (16), and it is well known that DAQ can modify cysteine residues of proteins, resulting in the formation of quinoproteins (16, 19, 30). It has been also reported that the free-radical scavengers NAC and ascorbic acid inhibit DA oxidation (31). In the present study, anti-oxidants NAC and ascorbic acid inhibited the attenuation of LPS-induced NO production by DA in BV-2 cells (Fig. 4A). In addition, DA concentration-dependently increased the quinoprotein level, and this increase was significantly inhibited by both NAC and ascorbic acid in BV-2 cells (Fig. 5A and B). Furthermore, tyrosinase accelerated the inhibitory effect of DA on LPS-induced NO production (Fig. 4D). These results suggest that DAQ generated by DA oxidation attenuated LPS-induced NO production in BV-2 cells.

It has been reported that DAQ conjugates with cysteine residues of several proteins such as tyrosine hydroxylase and tryptophan hydroxylase, and consequently inhibits their functions (17, 26). In the present study, DA increased the quinoprotein level (Fig. 5A) and decreased the LPS-induced expression of iNOS mRNA (Fig. 1D). These observations suggest that DAQ generated by the oxidation of DA modified cysteine residues of a certain protein that contributed to LPS-induced iNOS expression and thereby inhibited its function, resulting in the attenuation of LPS-induced NO production in BV-2 cells. As to the nature of such a protein, it has been reported that c-Jun N-terminal kinase (JNK), one of the 3 members of the mitogen-activated protein kinase family, plays an important role in TNF-α-induced iNOS induction and NO production in mouse macrophages (33, 34). We also previously reported that a selective JNK inhibitor attenuated LPS-induced iNOS expression and NO production in BV-2 cells (24). In addition, it was previously reported

Fig. 7. DA attenuated LPS-induced NO production in mouse microglial cells in primary culture. (A) Primary cultures of mouse microglial cells were treated with DA at the indicated concentrations for 24 h. Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significantly different from values without DA pretreatment (Sheffe's test). (B) Mouse microglial cells in primary culture were treated for 24 h with 10 μM DA in the absence or presence of 10 μM SCH-23390 or 10 μM sulpiride. Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference between bracketed values (Dunnett's test). (C) Mouse microglial cells in primary culture were treated with 10 μM CY 208-243 or 10 μM bromocriptine for 24 h. Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 3 independent experiments. $^{**}P < 0.01$, significantly different from values for LPS alone (Sheffe's test). (D) Mouse microglial cells in primary culture were treated for 24 h with 10 μM DA in the absence or presence of 10 mM NAC. Then the cells were treated with 0.1 μg mL^{-1} LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± s.e. obtained from 4 independent experiments. $^{**}P < 0.01$, significant difference between bracketed values (Dunnett's test).

that JNK has a conserved cysteine residue in its ATP-binding site and that the covalent binding of its residue by the selective inhibitor JNK–IN–8 inhibits the phosphorylation of c-Jun, a direct substrate of JNK (35). These observations suggest that DAQ generated by the oxidation of DA could inhibit JNK activity by modifying the cysteine residue in its ATP-binding site. Further studies are required to reveal the exact mechanism of DAQ-induced attenuation of NO production.

DA is chemically unstable and undergoes auto-oxidation to form DAQ and superoxide anion (15). This superoxide anion reacts rapidly with NO to produce peroxynitrite, which is a highly reactive free radical and mediates the toxicity of NO and the superoxide anion (36). Thus, the inhibition of NO production by DAQ might be important to protect the surrounding neuronal and glial cells from the toxicity of peroxynitrite. The overproduction of NO by activated microglial cells has been shown to induce neuronal injury in some models of neurodegenerative disorders and brain injury (11–13, 37, 38). In our study, DA attenuated not only LPS-stimulated but also IFN-γ and TNF-α-stimulated NO production in BV-2 cells (Fig. 1B and 6B), suggesting that DAQ would afford neuroprotection by inhibiting NO production by activated microglial cells. On the other hand, DAQ causes the death of some types of neuronal cells (39–41). Altogether, the effect of DAQ on neuronal survival could be both beneficial and detrimental, depending on the amount and the site of formation.

In conclusion, our results demonstrate that DA attenuated the NO production by LPS-stimulated microglial cells by reducing the induction of iNOS and that this reduced production was possibly mediated through the formation of DAQ.

Conflicts of interest

None of the authors have any conflicts of interest associated with this study.

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